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Award Number: W81XWH-04-1-0296

TITLE: Fish Oil Supplementation and Fatty Acid Synthase Expression in the Prostate: A Randomized Controlled Trial

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REPORT DATE: March 2008

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-03-2008		2. REPORT TYPE Final Addendum		3. DATES COVERED (From - To) 31 Mar 07 – 28 Feb 08	
4. TITLE AND SUBTITLE Fish Oil Supplementation and Fatty Acid Synthase Expression in the Prostate: A Randomized Controlled Trial				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0296	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Jackilen Shannon, Ph.D. E-Mail: shannoja@ohsu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Oregon Health and Science University Portland OR 97239-0396				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: One in seven men over the age of 60 will be diagnosed with prostate cancer. Elucidation of early cellular changes that may predict progression to prostate cancer and the identification of factors that may inhibit or reverse these cellular changes would be of great clinical significance. Alteration of the fatty acid synthase (FAS) pathway is an early cellular change that has recently come under investigation. Overexpression of the lipogenic enzyme FAS has been noted in several tumor and pre-cancerous tissue types, including prostatic intraepithelial neoplasia (PIN) and prostate cancer and has been suggested as an independent predictor of disease stage. Additionally, inhibition of FAS has been demonstrated to induce apoptosis and reduce cell proliferation in cancer cells. Fatty acid synthase expression in cancer and normal cells is regulated by the transcription factor sterol regulatory element binding protein 1c (SREBP-1). The up-regulation of SREBP-1 in tumor cells results in increased FAS expression and fatty acid synthesis. Research in normal cells has demonstrated that dietary supplementation with polyunsaturated fatty acids (PUFA), particularly omega-3 fatty acids, <u>inhibits SREBP-1 activation, resulting in a decreased transcription of FAS.</u>					
15. SUBJECT TERMS Prostate Cancer; Lipid Metabolism, Clinical Trial; Omega-3 Fatty Acids					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

SPECIFIC AIMS:

The protocol has been modified to describe addition of a green tea and green tea plus placebo arm. Based on discussion with the Project Officer (Julie Wilberding) it was determined this change to protocol does not affect the aims supported by DOD for the fish oil only trial. Thus no changes were made to the aims, Statement of Work or primary outcomes for the DOD funded trial. The two additional green tea arms are funded through a separate source (NCCAM), thus, in the following annual report we will not discuss the green tea portion of this trial.

We are conducting a double-blind, placebo-controlled, randomized intervention study to evaluate the effects of Fish Oil (FO) supplementation use on markers of lipid metabolism in prostate tissue samples. The primary endpoints of this trial are fatty acid synthase expression, caveolin-1 expression, changes in lipid raft fractions in the plasma membrane and cell proliferation (Ki-67 expression) in benign, pre-neoplastic and neoplastic prostate tissue. The secondary endpoints include measuring the expression of SREBP-1, a transcription factor for fatty acid synthase, cell death (apoptotic fraction using TUNEL), red blood cell (RBC) fatty acid concentration and change in PSA. Subjects are men from the Portland VA Medical Center (PVAMC), the Oregon Health and Science University (OHSU) and Kaiser Permanente Northwest (KPNW) urology clinics who are scheduled for a repeat biopsy. These men will have had an initial negative biopsy yet are still considered at high risk due to continued elevated prostatic specific antigen (PSA >4µg/dl), are positive for PIN, have suspicious findings by DRE or TRUS, or other clinical finding. Approximately 5 men per month over 24 months will be recruited and randomized to receive three months of either fish oil capsules (treatment 1) or olive oil (placebo) capsules (treatment 2). Potential confounding variables are assessed through completion of a comprehensive diet history questionnaire and risk factor questionnaire, assessment of pre and post-treatment PSA and surveillance of medication and supplement use. Compliance will be assessed using pill count and evaluation of RBC fatty acid concentrations. While this study population is limited to men at high risk of disease, the results may be more broadly generalizable to any man considered at risk of prostate cancer due to standard clinical indicators such as a PSA >4µg/ml.

BODY

During FY04 this award has supported study coordination, local and federal human subjects review, subject recruitment and data collection to address our primary aims. We also responded to the Oregon Health & Science University Cancer Institute's (OHSU CI) request for a voluntary audit. Unfortunately, because the audit was drawn out over the course of 9 months, it drastically affected last year's subject recruitment estimate. As a result of the audit, we developed a revised protocol that streamlined our eligibility criteria and added an extra layer of MD oversight prior to randomization. Last year, we failed to include the abstract in Box 14 of our annual DoD report. Please reference the full abstract as our first appendix this year.

HUMAN SUBJECTS REVIEW: Oversight for our protocol was transferred to USAMRMC HRPO on 1 September 2006; all minor modifications were reported to HRPO at the time of Continuing Review for all three sites. We utilized the HRPO Continuing Review Checklist and wrote corresponding explanation memos for these submissions to HRPO. The OHSU CI audit and its ensuing changes were submitted separately to the DOD as a major modification as well as a part of each site's continuing review. In response to the

findings of the OHSU CI audit, we submitted clarifications to the protocol to ensure that we continue to recruit and collect the data in the spirit in which the protocol was conceived and which will answer the scientific questions proposed.

The DOD approved modifications for all three sites throughout the year. For log number A-12538.a (PVAMC), the DOD approved the addition of the green tea arm, which was approved by the PVAMC IRB on 4/11/2007 (DOD approval: 6/22/2007). The DOD has not yet approved PVAMC's Continuing Review (PVAMC approval 4/11/2007). For log number A-12538.b (OHSU), the DOD approved the continuing review submission (DOD approval: 9/20/2007), which included correspondence between the OHSU CI auditors and the PI as well as changes to the protocol per OHSU CI audit. For log number A-12538.c (KPNW), the DOD approved the annual report on 11/1/2007 (KPNW approved 8/15/2007). This annual report included the OHSU CI audit reported as a protocol deviation.

STUDY COORDINATION: Ms. Cox remains the primary staff responsible for patient contact and recruitment procedures as well as on-going contact with collaborating clinicians. However, we welcome Ms. Courtney Maxcy who will soon become the primary study coordinator for the Fish Oil study. Because of our delayed recruitment over the past year, it has been imperative to create new, more pro-active and aggressive recruitment methods with our collaborating clinicians. Ms. Cox and Ms. Maxcy visit one of the two Kaiser clinics every week to maintain relationships with the clinicians, attend the OHSU clinic weekly and are working closely with the PVAMC clinician and his nurse to increase recruitment. All team members are involved in brainstorming new recruitment ideas; Ms. Palma designed a new, more eye-catching advertisement for this study, Ms. Farris also works directly with the PVAMC clinician to kick-start recruitment. In response to a Cancer Institute press release, the study was highlighted in several local papers and on the first page of the local Asian Reporter. There were also multiple radio announcements about the study. Ms. Farris currently retains primary responsibility for human subjects' paper work, continuing review documents and maintains ongoing contact with Johanna Kidwell (CDMRC). Senior research assistant, Ms. Amy Palma, is also available and trained to make recruitment phone calls to potential subjects, conduct visits, record data and complete all paperwork on each participant. All study staff have been trained on how to collect prostate biopsy cores from subjects, which are stored in a -80°C freezer.

PROGRESS TO DATE: As stated above, recruitment and enrollment onto the study ceased on 12/12/2006, per OHSU Cancer Institute (CI) audit. Due to the identification of multiple administrative deviations it was necessary to work with the OHSU CI, and with Oregon Clinical and Translational Research Institute's (OCTRI) Research Support and Services team to revise the full protocol such that the written protocol adequately reflects the intent of the trial. This revised and greatly simplified protocol was reviewed in April 2007, gaining IRB approval from OHSU on 7/5/2007 and PVAMC on 7/25/2007. We submitted these changes to KPNW at the time of annual review, which, as mentioned above, was approved on 8/15/2007. In addition, all research study staff have undergone additional clinical trials training and we have requested and received two voluntary quality assurance audits from the OCTRI Research Support and Services team and the solid tumors group within OHSU CI. Following final approval of the revised protocol and re-opening of recruitment we have worked diligently with the clinicians (as previously described) to enhance recruitment. In the past 7 weeks we have successfully enrolled 5 men into the trial. We are now averaging recruitment of approximately 3 to 4 new

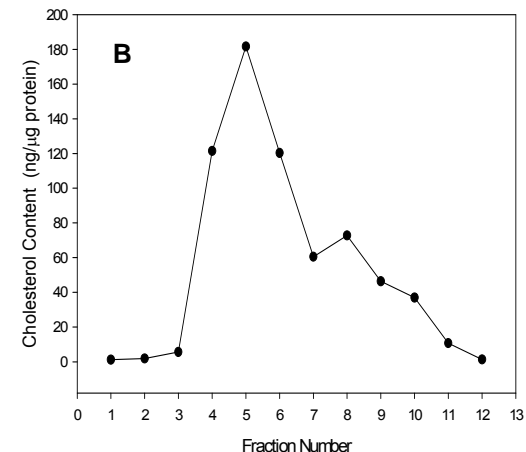
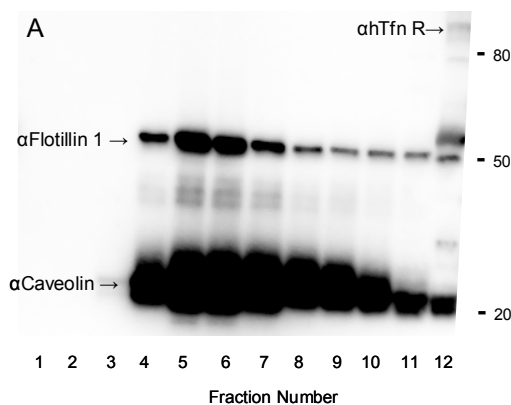
subjects per month, and believe that our numerous efforts in revitalizing this trial are now paying off with enhanced recruitment.

In addition, over the past year, we have continued work to optimize our laboratory procedures for estimating lipid raft and total cholesterol content within the prostate biopsy specimens. Lipid raft fractions were successfully prepared from surgical prostate samples using the methods described by Gebreselassie D and Bowen WD (1) and Martens et al (2) using discontinuous sucrose gradients to isolate the Triton X-100 insoluble raft fractions. The total cholesterol in density-gradient fractions will be determined using the Amplex Red Cholesterol Kit (Invitrogen, Molecular Probes, OR, USA). The assay was performed according to manufacturer's instructions. Triplicates of each raft prep fraction (10 μ l) were used in the assay and cholesterol standards were made to reflect sucrose/buffer content of each sample. The cholesterol concentration using this method was confirmed with liquid chromatography-mass spectroscopy (LC/MS) (3).

Western Blotting for proteins in lipid raft preparations will be done using a 20 μ l sample from each gradient fraction. Blots were probed with antibodies against flotillin 1, caveolin, and human transferring receptor to identify raft and nonraft fractions.

Initial studies were performed with prostate tissue removed during surgical procedures. Using these samples it was possible to remove the majority of the connective tissue and thus obtain a samples that could be homogenized reproducibly in the initial step with a Potter-Elvehjem Teflon-glass homogenizer in 25 mM MES, pH 6.5, with 150 mM NaCl (MBS) and 1% Triton X-100 (v/v). We found that in order to obtain a reproducible raft preparation we needed to start with about 100 mg of prostate tissue. After the initial homogenization and solubilization we were able to obtain a protein concentration of about 20 mg/ml. A representative raft preparation using these conditions is in Fig. 1.

Figure 1. Lipid raft cholesterol to protein ratio in human prostate. A) Flotillin1 and caveolin mark the lipid raft prep fractions (4-7). Frozen human prostate tissue was homogenized and subjected to centrifugation in a sucrose density gradient. Proteins were separated on a 4-12% gradient polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoblotting was performed with antibodies against human transferrin receptor, caveolin, and flotillin followed with secondary antibodies conjugated to horseradish peroxidase. Bands were visualized with chemiluminescence. B) The amount to cholesterol (ng/ μ g protein) in human prostate lipid raft fractions. Cholesterol was measured using Amplex Red Cholesterol Assay Kit from Molecular Probes according to established manufacturer's protocols. Protein was precipitated using the Pierce Compat-Able Protein Assay Preparations Reagent Set and



measured with Pierce BCA Protein Assay according to manufacturer's instructions.

When we attempted to use this protocol with two different needle stick biopsy samples we were not able to obtain detectable raft fractions. As a result, we assessed the possibility of using the needle stick biopsy samples for total cholesterol analysis using the Amplex Red Cholesterol Kit. Biopsy samples were homogenized directly in the assay buffer and a sample removed for protein analysis. We removed different amounts of total protein and found that 10 to 20 µg of total protein provided a reliable and reproducible cholesterol determination. In 2 different biopsy samples we found a range of 5 to 20 ng cholesterol/µg of protein. As a result, while we will not be able to assess raft fractions from the biopsy samples we will be able to determine the total cholesterol content and thus determine if the statin treatment altered the overall tissue concentrations of cholesterol.

KEY RESEARCH ACCOMPLISHMENTS: Immunohistochemistry for fatty acid synthase (FAS) and sterol regulatory element binding protein (SREBP-1) in the biopsy samples will occur in our final year of funding to allow for batching of the work and to ensure that the technicians are unaware of subject status when reviewing the samples. However, methods for quantitation of the cholesterol component of lipid rafts have been fully developed and tested in existing non-study tissue specimens.

REPORTABLE OUTCOMES: None to date

CONCLUSIONS: The primary outcome of the fourth year was the development of a more streamlined and compliant research protocol and enhancement of our recruitment methods. Through this process our investigative team has gained a great deal of experience in the development and conduct of a clinical trial in prevention. This is proving to be a great asset both to our study team and to the campus as a whole, as this type of translational prevention trial is new and previously there had been no local guidance from clinical trial experts available. We are continuing to recruit from the OHSU and PVAMC urology clinics

as well as two separate KPNW urology clinics. Recruitment from these additional sites not only improves our chances of reaching our accrual target, but also allows us to recruit from a more representative group of subjects. To date we have had no serious adverse events, and have only lost one individual post-randomization.

Table 1. DOD RCT Recruitment History

FISH OIL TRIAL RECRUITMENT: JANUARY 2005–MARCH 2008	
Fish Oil Participant Status	
Total patients eligible: 69	N (% of all eligible)
Enrolled in trial	37 (53.6)
Refused: gas / travel distance	6 (8.6)
Refused: other reasons	24 (34.7)
Screen fails	1 (1.0)
Administratively withdrawn	1 (1.0)
Identified potential patients awaiting eligibility determination	4
Total patients ineligible: 30	N (% of all ineligible)
Current statin use	8 (26.6)
Fish oil or Warfarin use	6 (20.0)
Other Criteria	12 (40.0)
Unknown	4 (13.3)
Number of patients introduced to study:	103

REFERENCES

1. Gebreselassie D, Bowen WD. Sigma-2 receptors are specifically localized to lipid rafts in rat liver membranes. *Eur J Pharmacol* 2004;493(1-3):19-28.
2. Martens JR, Navarro-Polanco R, Coppock EA, Nishiyama A, Parshley L, Grobaski TD, et al. Differential targeting of Shaker-like potassium channels to lipid rafts. *J Biol Chem* 2000;275(11):7443-6.
3. Brady JD, Rich TC, Le X, Stafford K, Fowler CJ, Lynch L, et al. Functional role of lipid raft microdomains in cyclic nucleotide-gated channel activation. *Mol Pharmacol* 2004;65(3):503-11.

ABSTRACT

Objectives: A number of complementary and alternative medicine (CAM) compounds, including omega-3 fatty acids (ω -3 FA) and green tea catechins have been hypothesized to reduce prostate cancer risk. However, it is important to understand the biologic mechanism underlying how they may function to slow or inhibit prostate carcinogenesis. One particular biologic pathway, which may be altered by both ω -3 FA and the specific green tea catechin, epigallocatechin-3-gallate (EGCG), is the fatty acid synthase (FAS) pathway. FAS, a lipogenic multienzyme that catalyzes the final step in de novo fatty acid synthesis, has been shown to be highly expressed in prostate carcinogenesis, and has been correlated with greater disease severity. Both EGCG and ω -3 FA have been shown in vitro and in vivo to inhibit this overexpression of FAS.

Plan: The primary objective of our proposal is to elucidate in men at high risk for prostate cancer, a potential biologic mechanism whereby EGCG, alone or in combination with ω -3 FA, may alter cellular composition and growth, thereby reducing overall risk of prostate cancer. In the proposed Clinical and Translational Research Center sponsored, randomized, placebo-controlled study we will evaluate the independent and joint effect of EGCG and EGCG plus ω -3 FA on FAS expression, FAS activity, cell proliferation and apoptosis in benign, pre-neoplastic and neoplastic prostate tissue of men undergoing repeat prostate biopsy.

Methods: We propose to conduct a placebo-controlled randomized intervention study of the effects of a fish oil and green tea supplement on FAS and sterol regulatory element binding protein-1 (SREBP-1) expression, cell proliferation and apoptosis in prostate tissue of men considered to be at elevated risk of prostate cancer. Patients eligible for this study will be men with an initial negative biopsy but that are considered at high risk and that are scheduled for a repeat biopsy. Approximately 72 men per year over two years will be recruited and randomized to receive three months of either (1) double placebo, (2) Fish Oil + placebo, (3) green tea + placebo, and (4) Fish Oil + green tea. While this study population includes only men who undergo repeat prostate biopsies, the results may still be generalizable to any men considered at risk of prostate cancer due to standard clinical indicators such as an elevated prostatic-specific antigen (PSA). All consenting patients will complete pre-intervention diet history and risk factor questionnaires, provide 20ml blood specimens (pre- and post-intervention), a urine specimen (pre- and post-intervention) and will be asked to provide 2 additional biopsy cores at post-intervention. Patients will be randomized to receive 3 capsules daily of either 1) ethyl esters of eicosapentaenoic acid (EPA) (20:5 n-3) and docosahexaenoic acid (DHA) (22:6 n-3), 2) green tea extract of 75% decaff (active treatments) or 3) to supplementation with ethyl esters of olive oil (matching fish oil placebo) or dicalcium phosphate (matching green tea placebo) for a total of 3 months. FAS and SREBP expression in prostate biopsy specimens will be determined at pre and post-intervention, markers of cell proliferation (Ki-67), apoptosis (TUNEL) and lipid raft fractions will be determined in post-intervention biopsy specimens.

Findings to Date: The primary outcome of the third year was our success in patient recruitment and the addition of a green tea and green tea plus fish oil arm to this trial. We have recruited a total of 33 men.

MeSH terms: Prostate Cancer; Lipid Metabolism; Clinical Trial; Omega-3 Fatty Acids

CLINICAL TRIAL STUDYING THE EFFECTS OF FISH OIL AND GREEN TEA IN PROSTATE CANCER PREVENTION



A Research Study

No cost to you. You will be compensated for travel to your first visit, whether you join the study or not. Study supplements are provided at no cost to you. **You may or may not benefit from being in this study.** However, by serving as a subject, you may help us learn how to benefit future patients.

How Do I Learn More About the Study?

Contact the Study Coordinator:
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Jackilen Shannon, PhD
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VA Clinician:
Mark Garzotto, MD
OHSU Clinician
Mitchell Sokoloff, MD

Who May Participate?

- Your doctor has recommended a repeat prostate biopsy
- You have never been diagnosed with prostate cancer
- You are not taking warfarin (Coumadin)
- You do not have a history of ventricular tachycardia or ventricular fibrillation
- Additional eligibility will be assessed during a brief telephone call

VAMC IRB #04-0303
OHSU IRB #1117



What Is Involved?

- Take fish oil or fish oil placebo supplements AND green tea or green tea placebo supplements for 3 months
- Four (4) blood samples
- Two (2) urine samples
- Diet History Questionnaire
- Two (2) prostate biopsy cores (during biopsy appointment)
- Total five (5) visits (2 in person and 3 over the phone)

